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Hemoglobin Binding of Aromatic Amines: Molecular Dosimetry and Quantitative Structure–Activity Relationships for *N*-Oxidation

by Gabriele Sabbioni[1]

Aromatic amines are important intermediates in industrial manufacturing. *N*-Oxidation to *N*-hydroxyarylamines is a key step in determining the genotoxic properties of aromatic amines. *N*-Hydroxyarylamines can form adducts with DNA, with tissue proteins, and with the blood proteins albumin and hemoglobin in a dose-dependent manner. The determination of hemoglobin adducts is a useful tool for biomonitoring exposed populations. We have established the hemoglobin binding index (HBI) [(mmole compound/mole hemoglobin)/(mmole compound/kg body weight)] of several aromatic amines in female Wistar rats. Including the values from other researchers obtained in the same rat strain, the logarithm of hemoglobin binding (logHBI) was plotted against the following parameters: the sum of the Hammett constants ($\Sigma\sigma = \sigma p + \sigma m$), p$K_a$, log$P$ (octanol/water), the half-wave oxidation potential ($E_{1/2}$), and the electronic descriptors of the amines and their corresponding nitrenium ions obtained by semi-empirical calculations (MNDO, AM1, and PM3), such as atomic charge densities, energies of the highest occupied molecular orbit and lowest occupied molecular orbit and their coefficients, the bond order of C–N, the dipole moments, and the reaction enthalpy [MNDOHF, AM1HF or PM3HF = Hf(nitrenium) − Hf(amine)]. The correlation coefficients were determined from the plots of all parameters against log HBI for all amines by means of linear regression analysis. The amines were classified in three groups: group 1, all *para*-substituted amines (maximum, n = 9); group 2, all amines with halogens (maximum, n = 11); and group 3, all amines with alkyl groups (maximum, n = 13). For the amines of group 1, logHBI correlates with $\Sigma\sigma$, AM1HF, $E_{1/2}$, the pK_a, and the log$P$ with r = 0.84, 0.73, 0.72, −0.69 and 0.50, respectively. For the amines of group 2, logHBI correlates with pK_a, $\Sigma\sigma$, MNDOHF, $E_{1/2}$, and log$P$ with r = 0.81, −0.80, −0.55, −0.46, and −0.20, respectively. For the amines of group 3, logHBI correlates with with $E_{1/2}$, PM3HF, Σs, pK_a, and log$P$ with r = 0.92, 0.89, 0.75, 0.19 and 0.12, respectively. This investigation shows for a large variety of aromatic amines the bioavailability of *N*-hydroxyarylamine (the genotoxic metabolite) and the utility of electronic descriptors for prediction of *N*-oxidation.

Introduction

Aromatic amines are important intermediates in industrial manufacturing. *N*-oxidation to *N*-hydroxyarylamines is a key step determining the genotoxic properties of aromatic amines. *N*-Hydroxyarylamines can form adducts with DNA, with tissue proteins, and with the blood proteins albumin and hemoglobin in a dose-dependent manner (*1,2*). *N*-Hydroxyarylamines are oxidized in erythrocytes to nitrosoarenes, which react with the β-93 cysteine of hemoglobin. The resulting *N*-hydroxy sulfinamide then rearranges to the more stable sulfinic acid amide (*1,3*). Recently it has been found that 4-aminobiphenyl is mainly oxidized by cytochrome P-450 IA2 and that high cytochrome P-450 IA2 expression correlates with hemoglobin binding (*3*). Furthermore, humans with the fast-oxidative phenotype have higher hemoglobin adduct levels. Therefore, hemoglobin adducts should be an appropriate dosimeter for *N*-hydroxyarylamines in biological systems. The main goal of our work is to determine the bioavailability of the potential ultimate carcinogen—the hydroxyarylamine—for a large array of substituted aromatic amines by measuring the amount of hydrolyzable hemoglobin adducts in rats.

Materials and Methods

Amines of the highest available purity were obtained from Riedel-de Haen (Selze, Germany), Aldrich (Steinheim, Germany), Fluka (Ulm, Germany), and Merck (Darmstadt, Germany). The purity of the amines was checked by GC–MS [Hewlett Packard instrument 5988, DB 1701 column (10 m × 0.32 mm × 1 μm)]. For each compound, two female Wistar rats were given 0.5 mmole/kg of amine by gavage. The rats were killed 24 hr later. The hemoglobin was isolated, hydrolyzed in 0.1 M NaOH, and extracted in hexane as described previously (*4,9*). The hexane fraction was analyzed by GC–MS with electron impact ionization in the single ion mode. Structure identification was based on the retention time and on the mass spectrum or the ratio of the main mass fragments. Amines with small hemoglobin binding (HBI $\leq$ 1) were derivatized with pentafluoropropionic acid anhydride and analyzed by GC–MS. To establish whether

[1]Institute of Toxicology, University of Würzburg, Versbacherstrasse 9, D-8700 Würzburg, Germany.

Table 1. Hemoglobin binding of aromatic amines.[a]

Group 1		Group 2		Group 3	
Compound	HBI	Compound	HBI	Compound	HBI
4-Methylmercaptoaniline	3.8 ± 0.5	2-Chloroaniline	0.5 ± 0.1	2-Methylaniline (*2*)	4.0 ± 1.0
4-Methylaniline (*2*)	4.3 ± 1.0	3-Chloroaniline (*4*)	12.5 ± 2.0	2-Ethylaniline	5.1 ± 1.1
4-Ethylaniline	5.8 ± 1.6	4-Chloroaniline (*2*)	569.0 ± 46.0	3-Methylaniline (*2*)	4.9 ± 0.2
Aniline (*2*)	22.0 ± 3.0	2,4-Dichloroaniline	0.6 ± 0.2	3-Ethylaniline	12.7 ± 1.5
4-Fluoroaniline	33.0 ± 9.0	2,6-Dichloroaniline	—	4-Methylaniline (*2*)	4.3 ± 1.0
4-Iodoaniline	296.0 ± 20.0	3,4-Dichloroaniline (*2*)	9.0 ± 2.0	4-Ethylaniline	5.8 ± 1.6
4-Bromoaniline	341.0 ± 14.0	3,5-Dichloroaniline (*4*)	0.6 ± 0.1	2,4-Dimethylaniline (*2*)	2.3 ± 1.0
4-Chloroaniline (*2*)	569.0 ± 46.0	2,3,4,5,6-Pentachloroaniline (*4*)	—	2,5-Dimethylaniline	7.3 ± 1.0
4-Trifluoromethylaniline	148.0 ± 6.0	3-Chloro-4-fluoroaniline	30.7 ± 2.4	2,6-Dimethylaniline	1.1 ± 0.3
		2,4-Difluoroaniline	32.0 ± 6.0	3,4-Dimethylaniline	0.7 ± 0.3
		4-Fluoroaniline	33.0 ± 9.0	3,5-Dimethylaniline	14.0 ± 1.9
		4-Iodoaniline	296.0 ± 20.0	2,4,5-Trimethylaniline (*2*)	0.7 ± 0.2
		4-Bromoaniline	341.0 ± 14.0	2,4,6-Trimethylaniline	0.2 ± 0.04

HBI, hemoglobin binding index [mmole (compound)/mole Hb]/[mmole (compound)/kg(body weight)].
[a] Reference numbers in parentheses where applicable.

the aromatic amines recovered from the alkaline hydrolysis were covalently bound, all samples were also extracted with hexane at neutral pH and analyzed by GC–MS.

Results and Discussion

The most hemoglobin binding (Table 1) was obtained with a halogen or a trifluoromethyl group in the *para* position. A chloro atom in the *ortho* position reduces the formation of hemoglobin adducts drastically (1000-fold, for 2-CA [chloroaniline] compared to 4-CA). This is not a result of the *para* position being free for hydroxylation because 2,4-DCA (dichloroaniline) has an HBI equal to that of 2-CA. An additional *ortho* chloro atom as in 2,6-DCA or PCA (pentachloroaniline) abolishes hemoglobin binding. A chlorine in the *meta* position decreases hemoglobin binding by a factor of 45 (3-CA compared with 4-CA). An additional chlorine in the *meta* position lowers the HBI further (20-fold).

For the methyl- or ethyl-substituted amines, we found the following relationships: all alkyl-substituted amines have a lower HBI than aniline. Only the HBI of 3,5-DMA (dimethylaniline) is comparable with that of aniline. The HBI of 3-EA (ethylaniline) is higher than that of 2-EA or 4-EA. This might be explained by the fact that the oxidation of alkyl groups in the *ortho* or *para* position to an amino group is facilitated compared to that of alkyl groups in the *meta* position. This trend is also seen in the dimethyl-substituted amines, where all amines show lower hemoglobin binding than the monosubstituted compounds except for 3,5-DMA. Two methyl groups in the *ortho* position, as in 2,6-DMA or 2,4,6-TMA (trimethylaniline), almost abolish hemoglobin binding.

In quantitative structure–activity studies, several descriptors are used. For our investigation we considered *a*) lipophilicity, *b*) electronic parameters that include ionization constants, the Hammett values, and the descriptors from molecular orbital calculations, and *c*) steric parameters. For our correlation analyses we included HBI values of other researchers that were determined in the same rat strain. For all amines, the coefficient of correlation between each parameter and the logarithm of HBI (logHBI) was determined by means of linear regression. The best correlation coefficient was found for PM3HF, with $r = 0.47$. Therefore, the amines were classified in three groups: group 1, all *para*-substituted amines (maximum, $n = 9$); group 2, all amines with halogens (maximum, $n = 11$); and group 3, all amines with alkyl groups (maximum, $n = 13$).

Correlation of logP with logHBI. We included all octanol-water partition coefficients (log*P*) available in the literature for aromatic amines. The best correlation of logP with logHBI was obtained for the amines of group 1, with $r = 0.50$ ($n = 8$), followed by group 2, with $r = -0.20$ ($n = 10$), and group 3, with $r = 0.12$ ($n = 11$). Analysis of the data with the Hansch equation also revealed poorly fitting curves. The partition coefficient alone is not sufficient to predict hemoglobin binding.

Correlation of the pKa with logHBI. The p*K*a and logHBI values correlate for group 2, with $r = 0.87$ ($n = 11$) (logHBI = $-2.452 + 1.127\,\mathrm{p}K_a$) for group 1, with $r = -0.69$ ($n = 9$), and for group 3, with 0.19 ($n = 13$). Thus, hemoglobin binding is indirectly proportional and directly proportional to the p*K*a values of the amines for *para*-substituted compounds and for halogen-substituted compounds, respectively.

Correlation of the Hammett Constants with logHBI. For multiply substituted compounds, the parameters were added. Group 1 correlates best with the logHBI, with $r = 0.84$ ($n = 9$), followed by group 2, with $r = -0.80$ ($n = 8$), and group 3, with $r = 0.75$ ($n = 6$). The Hammett constants are a useful tool for predicting hemoglobin binding of groups 1 and 2. The analysis is restricted by the lack of classical Hammett constants for *ortho*-substituted compounds.

Correlation of the Half-Wave Oxidation Potential with logHBI. Group 3 correlates with $r = 0.92$ ($n = 8$), followed by group 1 with $r = 0.72$ ($n = 8$), and group 2, with $r = -0.46$ ($n = 11$). This was the best correlation found for the alkyl-substituted amines.

Correlation of the Electronic Properties Calculated with MNDO, AM1, and PM3 with logHBI. We used semi-empirical molecular orbital calculations to obtain electronic parameters for all amines. The amines and nitrenium ion [the nitrenium ion is the best intermediate to describe the product distribution of cytochrome P-450 oxidations of amines (*5*)] were calculated without changing any of the default parameters with the three methods MNDO, AM1, and PM3 (*10*). We listed the heat of formation, the eigenvalues of all lowest occupied molecular orbits (LOMO) and highest occupied molecular orbits (HOMO), the coefficients of the molecular orbitals in the LOMO of the

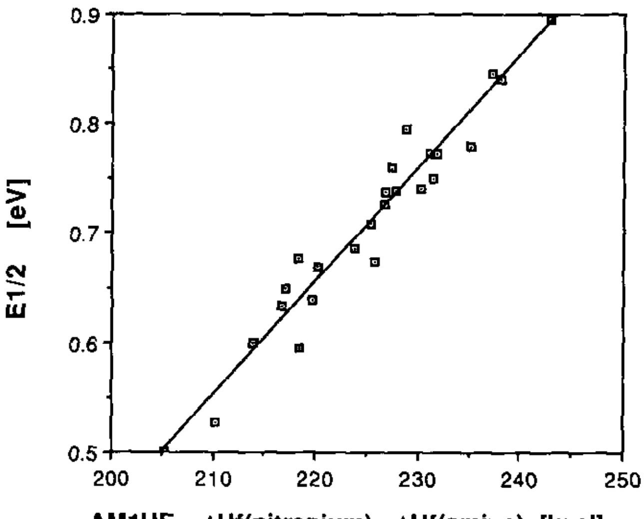

FIGURE 1. Correlation of $E_{1/2}$ with the reaction enthalpy of nitrenium formation (AM1HF) calculated with the program AM1 (MOPAC 6.0, Quantum Chemistry Program Exchange, Indiana University, Bloomington, IN) on a VAX 8810 (r = 0.97; $E_{1/2} = -1.658 + 0.0105$ AM1HF). All calculations were performed using the default parameter. The keyword PRECISE was used to increase the criteria for terminating all optimizations, electronic and geometric, by the factor 100. The starting geometries were created with the program PCMODEL (Serena Software, Bloomington, IN). The half-wave oxidation potential ($E_{1/2}$) of the aromatic amines was determined on an electrochemical detector, model 5100A, by ESA (Bedford, MA).

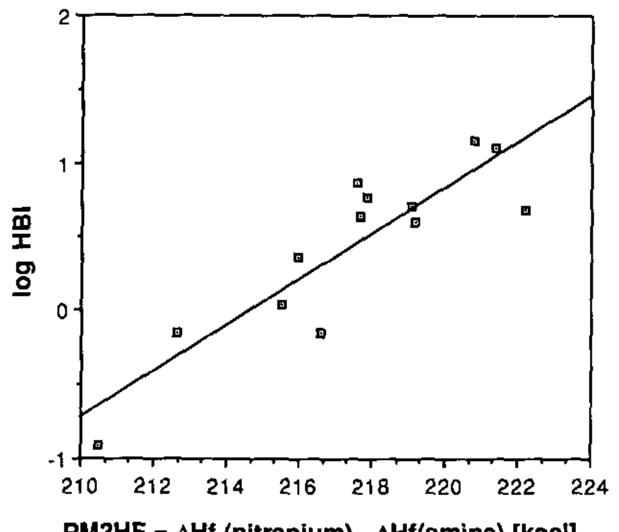

FIGURE 2. Correlation of logHBI (hemoglobin binding index) for methyl- or ethyl-substituted anilines (group 3) with the reaction enthalpy of nitrenium formation calculated with PM3 (PM3HF); r = 0.88; logHBI = -33.574 + 0.156 PM3HF.

nitrenium ions, and the molecular orbitals of the HOMO of the amines, the charge densities, the charge densities of the π-bonding system (pz), the bond length densities C–N, and all dipole moments. All values were plotted against logHBI. The heat of formation and the charges on the nitrogen atom gave the best results [the full data set and the comparison of the different calculation methods have been published elsewhere (9)]. The values of the calculated heat of formation (ΔHf) have to be expressed on a common basis in order to be compared. Th process of nitrenium formation was described by the equation $XRNH_2 \rightarrow XRNH^+ + 1H^+ + 2e^-$. MNDOHF, AM1HF, and PM3HF are the formal reaction enthalpies of this process [ΔHf(nitrenium)-ΔHf(amine)]. The loss of a proton and two electrons, which is the same for all calculated amines, and the entropy term are not included in these reaction enthalpies. Excellent correlations were found between MNDOHF or AM1HF and the Hammett constants or $E_{1/2}$ (Fig. 1). The calculated parameters can replace the Hammett parameters, which are not available for all amines. The values obtained with MNDO correlate well with AM1HF (r = 0.93) but poorly with PM3HF, especially with the values of group 2. The electron charge densities obtained with PM3 calculations for the nitrenium ions are different from the results obtained with MNDO and AM1. We used all the calculated values to find the best fit with logHBI. LogHBI correlates with MNDOHF [or AM1HF] of group 1 and group 3, with r = 0.71 [E0.73] (n =9) and 0.75 [0.84] (n = 13), respectively. By eliminating one oulier in group 1 (4-TFMA), the correlation increases to 0.96, (logHBI = -40.269 +0.189 MNDOHF). PM3HF correlates, with r = 0.88, with the logHBI of group 3. MNDO or AM1 calculations are a good predictive tool for amines of group 1. PM3 calculations predict well for group 3 (Fig. 2).

Are these structure–activity relationships found in rats transposable to other species? A larger set of amines has been studied regarding their capability to form methemoglobin (MetHb). Neumann (2) demonstrated that for several amines the extent of MetHb formation in rats and mice is similar to the extent of hemoglobin binding (r = 0.93). The structure–activity relationship for the MetHb response in cats (6) is similar to the HBI values in the present study. Compared to humans and mice, rats have two additional cysteine groups, which might react with nitrosoarenes (7), in the α-chain. Neumann (2) found similar structure–activity relationships in mice and rats, but 2–10 times lower hemoglobin binding in mice. Bryant et al. (8) demonstrated a comparable dose response for hemoglobin binding of 4-aminobiphenyl in rats and humans. Assuming that the human dose response to the amines (most of them from group 3) which has been found (8) is comparable to that of the rat, we estimated the daily exposure to alkylated amines to be between 0.014 and 23 μg per day per 70 kg nonsmoker. If these levels of exposure are realistic, the rat model could also be suitable for predicting effects in humans of amines other than 4-aminobiphenyl.

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